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(54) Titre : PROCEDE D'INHIBITION DE L'EXPRESSION D'UN GENE CIBLE ET MEDICAMENT DESTINE A LA THERAPIE D'UNE MALADIE TUMORALE

(54) Title: METHOD FOR INHIBITING THE EXPRESSION OF A TARGET GENE AND MEDICAMENT FOR TREATING A TUMOR DISEASE

(57) Abrégé/Abstract:
The invention relates to a method for inhibiting an expression of at least one target gene that impedes or prevents the apoptosis of a tumor cell, whereby at least one double-stranded ribonucleic acid (dsRNA) is introduced into the tumor cell whose strand S1 has a region, which is complementary to the target gene at least in areas and which is comprised of fewer than 25 consecutive nucleotides.

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Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(54) **Title:** METHOD FOR INHIBITING THE EXPRESSION OF A TARGET GENE AND MEDICAMENT FOR TREATING A TUMOR DISEASE

(54) **Bezeichnung:** VERFAHREN ZUR HEMMUNG DER EXPRESSION EINES ZIELGENS UND MEDIKAMENT ZUR THERAPIE EINER TUMORERKRANKUNG

(57) **Abstract:** The invention relates to a method for inhibiting an expression of at least one target gene that impedes or prevents the apoptosis of a tumor cell, whereby at least one double-stranded ribonucleic acid (dsRNA) is introduced into the tumor cell whose strand S1 has a region, which is complementary to the target gene at least in areas and which is comprised of fewer than 25 consecutive nucleotides.

(57) **Zusammenfassung:** Die Erfindung betrifft ein Verfahren zur Hemmung einer die Apoptose einer Tumorzelle hemmenden oder verhindernden Expression mindestens eines Zielgens, wobei mindestens eine doppelsträngige Ribonukleinsäure (dsRNA) in die Tumorzelle eingeführt wird, deren einer Strang S1 einen zum Zielgen zumindest abschnittsweise komplementären aus weniger als 25 aufeinanderfolgenden Nukleotiden bestehenden Bereich aufweist.

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Method for inhibiting the expression of a target gene and medicament for treating a tumor disease

The invention concerns a method for inhibiting the expression of at least one target gene in a cell, as well as a medicament for treating a tumor disease.

A method for inhibiting the expression of a target gene by means of a double-stranded oligoribonucleotide is known from WO 99/32619. The known method aims to inhibit the expression of genes in invertebrate cells. For this to occur, the double-stranded oligoribonucleotide must exhibit a sequence, consisting of at least 25 bases, that is identical to the target gene.

A method for inhibiting the expression of a target gene in a cell as well as a medicament is known from WO 00/44895. In this method, an oligoribonucleotide having a double-stranded structure (dsRNA) is introduced into the cell. One dsRNA strand exhibits a region consisting of a maximum of 49 sequential nucleotide pairs that is at least segmentally complementary to the target gene. The medicament contains at least one dsRNA for inhibiting the expression of a given target gene, whereby one dsRNA strand is at least segmentally complementary to the target gene.

It is known from Gautschi O. et al. (2001), J Natl Cancer Inst 93, pages 463 to 471, that an increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL is involved in the development and progress of many tumors. In vivo data generated in nude mice demonstrate that combined treatment with single-stranded antisense oligoribonucleotides directed against the expression of Bcl-2 and Bcl-xL genes could decrease the growth of tumors by approximately 50% to 60%. For that a treatment with 20 mg oligoribonucleotides per kilogram body weight and day was required. Due to the large quantity of oligoribonucleotides required the treatment is relatively expensive. Over and above that, the used single-stranded oligoribonucleotides can be broken down quickly in serum. The large quantity of oligoribonucleotides is required because an antisense

oligoribonucleotide must be introduced in the end into target cells in a quantity that is at least as large as the quantity of target-gene mRNA being there. This method merely achieved a decrease in growth, but did not shrink the tumors.

The task of the present invention is to remove these shortcomings in accordance with the state-of-the-art. In particular, a method and a medicament are to be disclosed by which the multiplication of tumor cells can be effectively and economically inhibited.

This task is solved by the features of in Claims 1 and 13. Advantageous embodiments result from Claims 2 to 12 and 14 to 26.

According to the provisions of the invention, a method is intended for inhibiting the expression of at least one target gene that inhibits or prevents apoptosis in a tumor cell, whereby at least one double-stranded ribonucleic acid (dsRNA) , whose strand S1 contains a region consisting of fewer than 25 sequential nucleotides that is at least segmentally complementary to the target gene, is introduced into the tumor cell. The term “target gene” is understood to mean the DNA strand of the double-stranded DNA in the tumor cell that is complementary to a DNA strand that serves as a matrix for transcription, including all transcribed regions. The target gene so is generally the sense strand. The strand S1 can thus be complementary to an RNA transcript that is formed during expression of the target gene, or to its processing products, e.g., an mRNA. DsRNA is present when the ribonucleic acid, consisting of one or two ribonucleic acid strands, exhibits a double-stranded structure. Not all dsRNA nucleotides must exhibit Watson-Crick base pairings. Single non-complementary base pairs in particular hardly interfere at all with the method. The maximum possible number of base pairs is the number of nucleotides in the shortest strand contained in the dsRNA. The term "introduced into" is taken to mean uptake in the cell. Uptake may occur by means of the cell itself. However, it may also be mediated by auxiliary agents or devices.

Surprisingly, it has been shown that using this method the multiplication of tumor cells can be inhibited with a considerably smaller quantity of oligoribonucleotides than is

possible when using oligonucleotides to achieve comparable results with traditional antisense technique. Over and above that, it is possible, using the method that is the subject of this invention, to induce apoptosis to such an extent in a population of tumor cells that not only the growth of the population is decreased, but also the total number of tumor cells. Implementation of the method with normal, i.e., non-transformed, cells induces no significant increase in the rate of apoptosis in comparison with a method carried out with a control dsRNA. Control dsRNA is dsRNA that exhibits no strand that is complementary to one of the genes present in the cells.

It has been shown to be particularly advantageous when at least one end of the dsRNA exhibits a single-stranded overhang consisting of 1 to 4, in particular of 2 or 3, nucleotides. In comparison to dsRNA without a single-stranded overhang at at least one end, such dsRNA demonstrates superior effectiveness in inhibiting expression of the target gene. Here, one end is a dsRNA region in which a 5'- and a 3'-strand-end is present. DsRNA consisting only of the strand S1 accordingly exhibits a loop structure and only one end. DsRNA consisting of the strand S1 and a strand S2 exhibits two ends. Here, one end is formed in each case by a strand end on the strand S1 and one on the strand S2.

The single-stranded overhang is preferably located at the 3'-end of the strand S1. This location of the single-stranded overhang leads to a further increase in the efficiency of the method. In one example, the dsRNA exhibits a single-stranded overhang at only one end, in particular that located at the 3'-end of the strand S1. In dsRNA that exhibits two ends, the other end is blunt, i.e., without overhangs. Such dsRNA has proven to be particularly stable in a variety of cell culture media, as well as in blood serum.

The complementary dsRNA region may exhibit 19 to 24, preferably 21 to 23, and particularly 22, nucleotides. DsRNA with this structure is particularly effective in inhibiting the target gene. The strand S1 of the dsRNA may exhibit fewer than 30, preferably fewer than 25, and particularly 21 to 24 nucleotides. The number of these nucleotides is concurrently the number of base pairs maximally possible in the dsRNA.

At least one end of the dsRNA can be modified in order to counter breakdown in the tumor cell or dissociation of the double-stranded structure. Furthermore, the bonding of the double-stranded structure that is effected by complementary nucleotide pairs may be increased by at least one, preferably two, further chemical bond(s). The chemical bond may be formed either by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, preferably van der Waals or stacking interactions, or by metal-ion coordination. It can also be formed by using purine analogues instead of purines in the double-stranded structure.

In a preferred embodiment of the method, the target gene is at least one gene belonging to the Bcl-2 family, in particular Bcl-2, Bcl-w, or Bcl-xL. It is also possible that several genes are target genes. Therefore, both Bcl-2 and Bcl-xL may be target genes. Inhibition of the genes of the Bcl-2 family is particularly good because their increased expression is linked to the development and growth of many tumor cells. The inhibition of several target genes is advantageous because there are tumor cells that express several anti-apoptotic genes.

The dsRNA preferably consists of a strand S2 having the sequence SEQ ID NO: 1 and the strand S1 having the sequence SEQ ID NO: 2; or of a strand S2 having the sequence SEQ ID NO: 3 and the strand S1 having the sequence SEQ ID NO: 4, in accordance with the attached sequence listing. Such dsRNA is particularly effective in inhibiting the expression of the Bcl-2 target gene. The tumor cell may be a pancreatic carcinoma cell. In order to introduce the dsRNA into the tumor cell, a micellar structure that surrounds the dsRNA, preferably a liposome, or a capsid that surrounds the dsRNA, may be used. In particular, the capsid may be a natural viral capsid, or a synthetic capsid produced by chemical or enzymatic means, or a structure derived from it.

Furthermore, the invention concerns a medicament for treating a tumor disease which contains at least one double-stranded ribonucleic acid (dsRNA) for inhibiting an expression of at least one target gene, whereby a strand S1 of the dsRNA exhibits a region consisting of fewer than 25 sequential nucleotides that is at least segmentally

complementary to a target gene. Here, the target gene is a gene whose expression inhibits or prevents apoptosis in tumor cells. The dosage of the medicament is to be such that expression of at least one target gene may be inhibited. Surprisingly, it has been shown that such a medicament may be used at very low dosages. A dosage of 5 mg dsRNA per kilogram body weight and day is sufficient to inhibit or completely suppress the expression of the target gene in the tumor cells. Side effects are largely avoided at such a low dosage.

Preferably, at least one end of the dsRNA exhibits a single-stranded overhang consisting of 1 to 4, particularly of 2 or 3 nucleotides. The single-stranded overhang may be located at the 3'-end of the strand S1. It is particularly advantageous if the dsRNA exhibits a single-stranded overhang at only one end, in particular that located at the 3'-end of the strand S1. It has been demonstrated that such dsRNA is particularly stable in the body. It is excreted or broken down in the blood more slowly than is dsRNA with single-stranded overhangs at both ends. This makes low dosages possible.

The complementary region may exhibit 19 to 24, preferably 21 to 23, and particularly 22, nucleotides. The strand S1 may exhibit fewer than 30, preferably fewer than 25, and particularly 21 to 24 nucleotides. In one implementation form, at least one end of the dsRNA is modified to counter breakdown in the tumor cells or dissociation. Bonding of the double-stranded structure that is effected by complementary nucleotide pairs may be increased by at least one, preferably two, further chemical bond(s).

The target gene is preferably at least one gene belonging to the Bcl-2 family, in particular Bcl-2, Bcl-w, or Bcl-xL. A medicament that exhibits a dsRNA specific for both the Bcl-2 target gene and for the Bcl-xL target gene is particularly efficient.

The dsRNA may consist of a strand S2 having the sequence SEQ ID NO: 1 and the strand S1 having the sequence SEQ ID NO: 2; or of a strand S2 having the sequence SEQ ID NO: 3 and the strand S1 having the sequence SEQ ID NO: 4, in accordance with the attached sequence listing. The tumor disease that can to be treated with this medicament

may be a pancreatic carcinoma. No sufficiently successful therapy for pancreatic carcinoma exists at present. At approximately 3%, the 5-year survival rate is the lowest of all carcinomas. The dsRNA may be present in the medicament either in solution or surrounded by a micellar structure, preferably by a liposome, or by a capsid. A micellar structure or a capsid can facilitate the uptake of dsRNA in the tumor cells. The medicament may exhibit a preparation that is suitable for inhalation, oral ingestion, or injection, particularly for intravenous or intraperitoneal injection, or for injection directly into tumor tissue. A preparation suitable for inhalation or injection can, most simply, consist of the dsRNA and a physiologically tolerated buffer, particularly a phosphate buffered saline solution. Surprisingly, it has been shown that dsRNA which is simply dissolved in such a buffer is taken up by the tumor cells and inhibits the expression of the target gene, without the dsRNA having had to be packaged in a particular vehicle.

In the following, examples of the invention will be explained on the basis of the figures. They show:

Figure 1 the apoptosis rate (in percent) of human pancreatic carcinoma cells YAP C, 120 hours after transfection with a dsRNA 1 that is complementary to a first sequence of the human Bcl-2 gene;

Figure 2 the apoptosis rate (in percent) of the YAP C cells, 120 hours after transfection with a dsRNA 2 that is complementary to a second sequence of the human Bcl-2 gene;

Figure 3 the apoptosis rate (in percent) of YAP C cells, 120 hours after transfection with a dsRNA 3 that is complementary to a sequence of the neomycin resistance gene.

The cells of the human pancreatic carcinoma cell line YAP C, which may be obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, (No. ACC 382), were cultured under constant conditions at 37°C, 5% CO_2 in RPMI 1640

medium (Biochrom Corp., Berlin) with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Human skin fibroblasts were cultured under the same conditions in Dulbecco's MEM with 10% FCS and 1% penicillin/streptomycin.

The double-stranded oligoribonucleotides used for transfections exhibit the following sequences, designated as SEQ ID No:1 to SEQ ID No:6 in the sequence listing:

dsRNA 1, which is complementary to a first sequence of the human Bcl-2 gene:

S2: 5'- cag gac cuc gcc gcu gca gac c-3' (SEQ ID NO: 1)
S1: 3'-cg guc cug gag cgg cga cgu cug g-5' (SEQ ID NO: 2)

dsRNA 2, which is complementary to a second sequence of the human Bcl-2 gene:

S2: 5'- g ccu uug ugg aac ugu acg gcc-3' (SEQ ID NO: 3)
S1: 3'-uac gga aac acc uug aca ugc cgg-5' (SEQ ID NO: 4)

dsRNA 3, which is complementary to a sequence of the neomycin resistance gene:

S2: 5'- c aag gau gag gau cgu uuc gca-3' (SEQ ID NO: 5)
S1: 3'-ucu guc cua cuc cua gca aag cg –5' (SEQ ID NO: 6)

Transfections were carried out in a 6-well plate with oligofectamine (Invitrogen Corp., Karlsruhe). 250,000 cells were placed in each well. Transfection of the double-stranded oligoribonucleotides was carried out in accordance with the protocol recommended by Invitrogen for oligofectamine (the data relate to 1 well of a 6-well plate):

10 μl of the double-stranded oligoribonucleotide (0.1-10 μM) was diluted with 175 μl cell culture medium without additives. 3 μl oligofectamine was diluted with 12 μl cell culture medium without additives, and incubated for 10 minutes at room temperature. The diluted oligofectamine was added to the already diluted double-stranded

oligoribonucleotides, mixed, and incubated for 20 minutes at room temperature. During this time, the cells to be transfected were washed once with cell culture medium without additives, and 800 μl of fresh cell culture medium was added. After that 200 μl of the described oligofectamine-dsRNA-mixture were added per well so that the end volume for the transfection was 1000 μl. This results in an end concentration of the double-stranded oligoribonucleotide of 1-100 μM. The transfection assay was incubated for four hours at 37°C. After that, 500 μl of cell culture medium with 30% FCS were added per well so that the end concentration of FCS was 10%. This assay was then incubated for 120 hours at 37°C.

After incubation, the supernatants were collected, the cells were washed with phosphate buffered saline (PBS), detached using trypsin, and centrifuged for 10 minutes at 100 g. The supernatant was discarded, and the pellet was incubated in the dark with hypotonic propidium iodide solution for 30 minutes at 4°C. Analysis followed using flow cytometry in an FACSCalibur fluorescence-activated cell sorter (BD GmbH, Heidelberg).

The double-stranded oligoribonucleotides dsRNA 1 and dsRNA 2 decrease the inhibition of apoptosis mediated by Bcl-2 in the human pancreatic carcinoma cells studied. No additional stimulation of apoptosis is required to induce or initiate apoptosis. The apoptosis rate rose in dependence on the incubation time. Figure 1 shows the result achieved with dsRNA 1 and Figure 2 that with dsRNA 2. Whereas untreated YAP C control cells and cells with which the described method for transfection was carried out without double-stranded oligoribonucleotides (mock-transfected cells) showed only 3.8% and 7.1% apoptossis after 120 hours of incubation, the apoptosis rate after 120 hours could be increased by transfection with 100 nM dsRNA to 37.2% for transfection with dsRNA 1 and 28.9% for transfection with dsRNA 2. Control transfection with dsRNA 3 led to a maximum apoptosis rate of 13.5%. This represents no significant increase when compared to mock-transfected cells, and proves the sequence specificity of the action of the dsRNA 1 and dsRNA 2.

As a control, skin fibroblasts as non-transformed cells were also transfected with dsRNA 1 and dsRNA 2. After 120 hours these cells showed no significant increase in apoptosis rate.

Patent Claims

1. Method for inhibiting the expression of at least one target gene that inhibits or prevents apoptosis in a tumor cell, whereby at least one double-stranded ribonucleic acid (dsRNA), whose strand S1 exhibits a region consisting of fewer than 25 sequential nucleotides that is at least segmentally complementary to the target gene is introduced into the tumor cell.

2. Method in accordance with Claim 1, whereby at least one end of the dsRNA exhibits a single-stranded overhang, consisting of 1 to 4, particularly of 2 or 3 nucleotides.

3. Method in accordance with one of the previous claims, whereby the single-stranded overhang is located at the 3'-end of the strand S1.

4. Method in accordance with one of the previous claims, whereby the dsRNA exhibits a single-stranded overhang only at one end, in particular that located at the 3'-end of the strand S1.

5. Method in accordance with one of the previous claims, whereby the complementary dsRNA region exhibits 19 to 24, preferably 21 to 23, and particularly 22, nucleotides.

6. Method in accordance with one of the previous claims, whereby the strand S1 exhibits fewer than 30, preferably fewer than 25, and particularly 21 to 24 nucleotides.

7. Method in accordance with one of the previous claims, whereby at least one end of the dsRNA is modified in order to counter breakdown in the tumor cell or dissociation.

8. Method in accordance with one of the previous claims, whereby the bonding of the dsRNA effected by complementary nucleotide pairs is increased by at least one, preferably two, other chemical bond(s).

9. Method in accordance with one of the previous claims, whereby the target gene is at least one gene belonging to the Bcl-2 family, particularly Bcl-2, Bcl-w, or Bcl-xL, or Bcl-2 and Bcl-xL are target genes.

10. Method in accordance with one of the previous claims, whereby the dsRNA consists of a strand S2 having the sequence SEQ ID NO: 1 and the strand S1 having the sequence SEQ ID NO: 2; or a strand S2 having the sequence SEQ ID NO: 3 and the strand S1 having the sequence SEQ ID NO: 4, in accordance with the attached sequence listing.

11. Method in accordance with one of the previous claims, whereby the tumor cell is a pancreatic carcinoma cell.

12. Method in accordance with one of the previous claims, whereby the dsRNA is introduced into the tumor cell by means of a micellar structure, preferably a liposome, that surrounds the dsRNA, or by a capsid that surrounds the dsRNA.

13. Medicament to treat a tumor disease, containing at least one double-stranded ribonucleic acid (dsRNA) for inhibiting an expression of at least one target gene that inhibits or prevents apoptosis in tumor cells, whereby a strand S1 of the dsRNA exhibits a region consisting of fewer than 25 sequential nucleotides that is at least segmentally complementary to the target gene.

14. Medicament in accordance with Claim 13, whereby at least one end of the dsRNA exhibits a single-stranded overhang, consisting of 1 to 4, particularly of 2 or 3 nucleotides.

15. Medicament in accordance with one of Claims 13 or 14, whereby the single-stranded overhang is located at the 3'-end of the strand S1.

16. Medicament in accordance with one of Claims 13 to 15, whereby the dsRNA exhibits a single-stranded overhang only at one end, in particular that located at the 3'-end of the strand S1.

17. Medicament in accordance with one of Claims 13 to 16, whereby the complementary region exhibits 19 to 24, preferably 21 to 23, particularly 22, nucleotides.

18. Medicament in accordance with one of Claims 13 to 17, whereby the strand S1 exhibits fewer than 30, preferably fewer than 25, and particularly 21 to 24 nucleotides.

19. Medicament in accordance with one of Claims 13 to 18, whereby at least one end of the dsRNA is modified in order to counter breakdown in the tumor cells or dissociation.

20. Medicament in accordance with one of Claims 13 to 19, whereby the bonding of the dsRNA effected by complementary nucleotide pairs is increased by at least one, preferably two, other chemical bond(s).

21. Method in accordance with one of Claims 13 to 20, whereby the target gene is at least one gene belonging to the Bcl-2 family, particularly Bcl-2, Bcl-w, or Bcl-xL, or Bcl-2 and Bcl-xL are target genes.

22. Medicament in accordance with one of the Claims 13 to 21, whereby the dsRNA consists of a strand S2 having the sequence SEQ ID NO: 1 and the strand S1 having the sequence SEQ ID NO: 2; or a strand S2 having the sequence SEQ ID NO: 3 and the strand S1 having the sequence SEQ ID NO: 4, in accordance with the attached sequence listing.

23. Medicament in accordance with one of Claims 13 to 22, whereby the tumor disease is a pancreatic carcinoma.

24. Medicament in accordance with one of the Claims 13 to 23, whereby the dsRNA is present in the medicament in solution or surrounded by a micellar structure, preferably a liposome, or by a capsid.

25. Medicament in accordance with one of Claims 13 to 24, whereby the medicament exhibits a preparation that is suitable for inhalation, oral ingestion, or injection, particularly for intravenous or intraperitoneal injection, or for injection directly into a tumor tissue.

26. Medicament in accordance with Claim 25, whereby the preparation consists of the dsRNA and a physiologically tolerated buffer, particularly a phosphate-buffered saline solution.

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<223> Description of the synthetic sequence: antisense strand of a dsRNA that is complementary to a sequence of the neomycin resistance gene
<400> gcgaaacgau ccucauccug ucu 23
WO 02/055692 PCT/EP02/00151

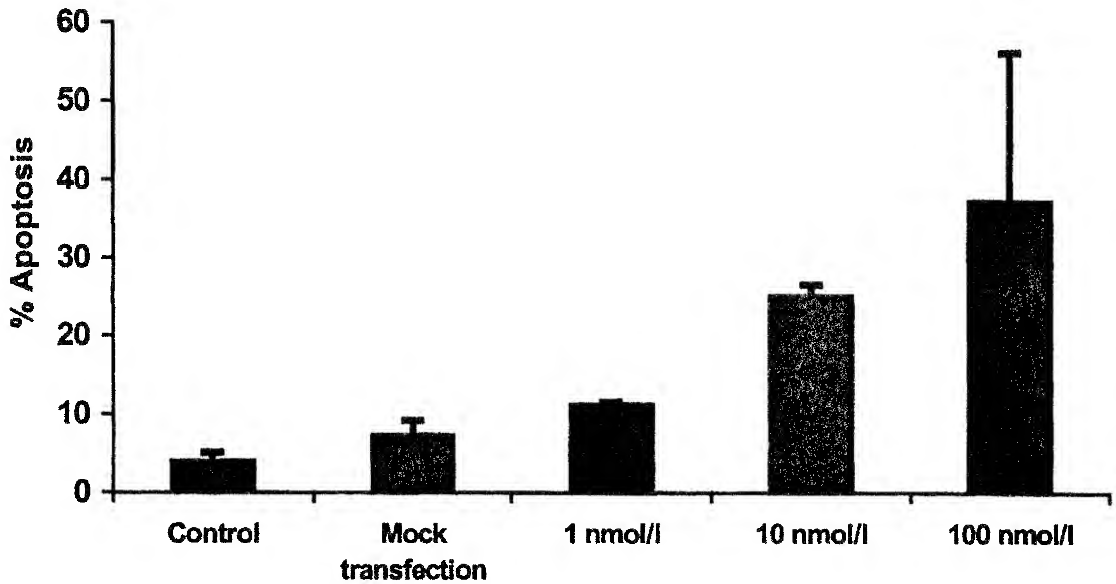
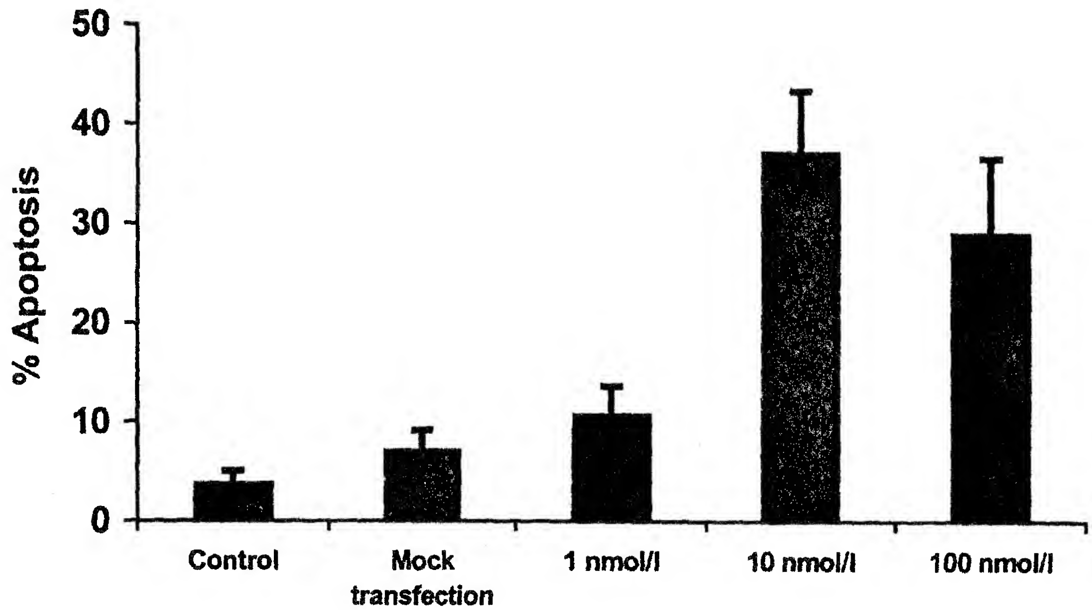

60
50
40
30
20
10
0
% Apoptosis
Control
Mock transfection
1 nmol/l
10 nmol/l
100 nmol/l

Figure 1



50
40
30
20
10
0
% Apoptosis
Control
Mock transfection
1 nmol/l
10 nmol/l
100 nmol/l

Figure 2

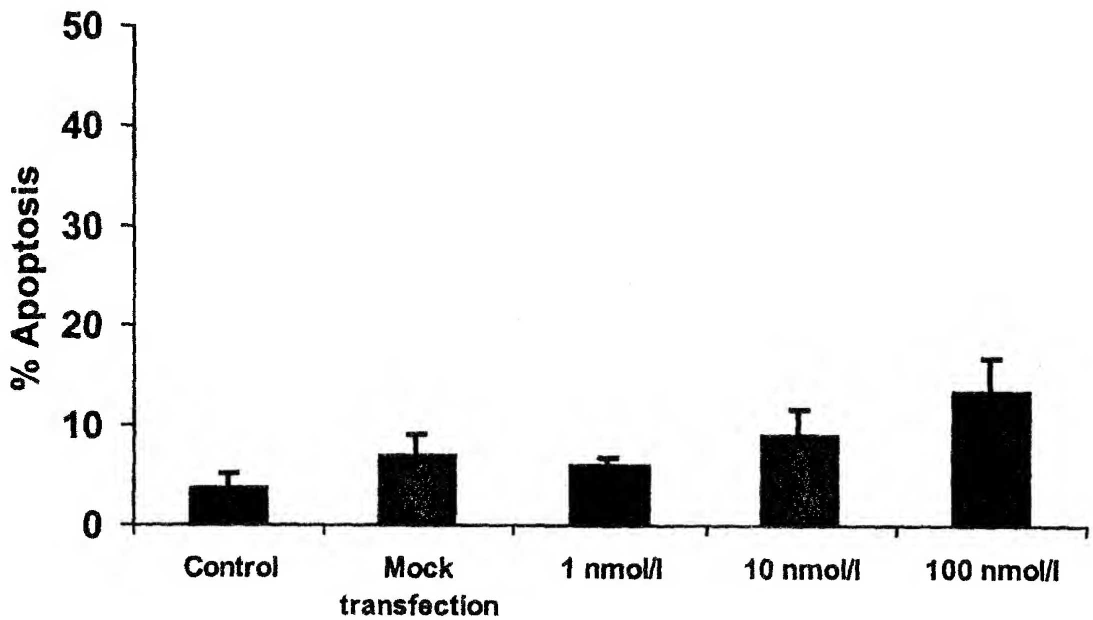

50
40
30
20
10
0
% Apoptosis
Control
Mock transfection
1 nmol/l
10 nmol/l
100 nmol/l

Figure 3